Image cytometry DNA ploidy analysis: correlation between two semi-automated methods

Janaina Salomon Ghizoni (1)
Marcelo Sperandio (2)
Claire Lock (3)
Edward W Odell (4)

1. PhD. Department of Oral Pathology & Medicine, University of South Santa Catarina (UNISUL), Avenida José Acácio Moreira, 787, CEP 88704-900 – Tubarão – SC, Brazil.

2. PhD. Department of Oral Pathology & Medicine, Sao Leopoldo Mandic Dental Institute and Research Center, Rua Jose Rocha Junqueira, 13, Campinas-SP, CEP 13045-755, Brazil

3. MSc. Head and Neck Pathology, Viapath LLC, Guy's Hospital, London SE1 9RT. United Kingdom

4. PhD FRCPath., Department of Oral Pathology King's College London. Guy's Hospital, London SE1 9RT. United Kingdom

Running title: DNA ploidy analysis: comparing two systems

Corresponding author: Marcelo Sperandio
Department of Pathology, São Leopoldo Mandic Medical & Dental Institute.
Rua José Rocha Junqueira, 13. Campinas, SP, Brazil, 13045-755.
Telephone: +55 19 3211 3600
e-mail: marcelo.sperandio@slmandic.edu.br

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Abstract

Background and objectives

Quantitation of cell DNA content, DNA ploidy, has been established as a research and prognostic technique for decades. A variety of instruments have been used despite only a few commercially available systems having established quality assurance and published outcome data. The aim of this study was to compare two automated systems.

Methods

Nuclear monolayers were obtained from 112 oral biopsies by enzyme digestion and Feulgen staining. These were scanned on both the Fairfield and the Ploidy Work Station (PWS) systems. The overall ploidy diagnosis, number of epithelial nuclei, coefficient of variation (CV) and 5c-exceeding rate (5CER) were compared by quantile-quantile plots, t-test, Wilcoxon and Spearman tests.

Results

The PWS system identified more nuclei ($p<0.0001$) at a lower CV ($P<0.0001$). Using the PWS system, fewer samples were classified as indeterminate. No difference between 5CER was found between systems ($p>0.54$). There was complete concordance between the two systems in terms of DNA ploidy diagnosis.

Conclusions

The PWS system is comparable to the Fairfield system for determination of DNA ploidy and has advantages that may lead to improved performance.

Introduction

The presence in tissues of cells with abnormal nuclear DNA content, or DNA aneuploidy, is an indicator of chromosomal instability. Despite the fact that chromosomal instability is not only a hallmark of cancer, but a cancer driver mechanism (Davoli, 2013) and an early event in carcinogenesis (Williams & Amon, 2009), detecting it by DNA ploidy analysis remains in relatively limited use. Until recently, it appeared to be of limited value.(Baak & Janssen, 2004; Hall, 2004)

DNA ploidy analysis is, however, proving increasingly useful in diagnosis, prognostication for malignant neoplasms and to predict malignant transformation in precancerous conditions. DNA ploidy analysis by image based cytometry predicts survival in colorectal carcinoma,(Hveem, 2014) malignant transformation in Barrett's oesophagus(Dunn et al., 2010) and in oral dysplasia.(Sperandio et al., 2013) These and other applications in gastrointestinal, breast, prostate, endometrial and other cancers have been reviewed recently.(Danielsen, Pradhan, & Novelli, 2015)

We have been using DNA ploidy analysis to predict malignant transformation in upper aerodigestive tract dysplasia for over 15 years. Prediction by routine histopathology remains the standard method, but is criticized as poorly reproducible(Brothwell et al., 2003; Karabulut, 1995; Warnakulasuriya, Reibel, Bouquot, & Dabelsteen, 2008) and poorly predictive.(Dost, Lê Cao, Ford, Ades, & Farah, 2014; Holmstrup, Vedtofte, Reibel, & Stoltze, 2006) As an alternative, DNA ploidy analysis performed by karyometry,(Abdel-Salam, Mayall, Chew, Silverman, & Greenspan, 1988) high-resolution flow cytometry(Brouns et al., 2012; Grassel-Pietrusky, Deinlein, & Hornstein, 1982) and image cytometry(Bradley et al., 2010; Tabor et al., 2003; Torres-Rendon, Stewart, Craig, Wells, & Speight, 2009) can identify lesions at risk. Of these methods, semi-automated image cytometry has several advantages. Specific areas of interest in the tissue can be selected, routine paraffin-embedded biopsy samples can be used, data collection is rapid and cells with DNA content exceeding 5c can be detected. In our hands the technique is strongly predictive of malignant transformation in oral mucosa(Marcelo Sperandio

et al., 2013) and in specific oral potentially malignant disorders.(Klanrit et al., 2007; Marcelo Sperandio et al., 2016)

Wider adoption of the technique has been hampered by lack of instruments with published outcome and quality assurance data. This is important because apparently similar systems capable of accurate DNA quantitation (Bradley et al., 2010; M Sperandio, 2013) may have differing ability when tested in outcome studies. Much of the published data on quality assurance and accuracy were based on the Fairfield system (previously from Fairfield Imaging, Tunbridge Wells, UK and subsequently Medical Solutions, Nottingham UK), which has not been available since 2005. However, instruments remain in use in several centres in Europe, generating considerable data in support of its effectiveness.(Crisp, Burton, Stewart, & Wells, 2003; Diwakar, Sperandio, Sherriff, Brown, & Odell, 2005; Dunn et al., 2010; Sperandio et al., 2013; Torres-Rendon et al., 2009) The system ceased to be available for commercial reasons and its software and computer platform became progressively obsolete. The Ploidy Work Station system (PWS; Room4 Group Ltd, East Sussex, UK) is a newer product available since 2005 and sharing some of the hardware specification of the Fairfield system and with updated imaging and software and designed by the originators of the Fairfield system at the Norwegian Radium Hospital. Many publications have been generated with the PWS system.(Mouradov, 2013; Pradhan et al., 2012; Pretorius et al., 2009)

The aims of this study were to test the PWS system against the Fairfield system to determine whether clinical treatment decisions based on the Fairfield system would be applicable to the PWS system, by assessing concordance of results and performance.

Materials and Methods

Sample selection

One hundred and twelve consecutive biopsy samples of oral mucosa taken for assessment of risk of malignant transformation were selected. The study was approved by the Guy's Hospital Research Ethics Committee and the use of material and data by the UK Patient Information Advisory Group [reference PIAG 4-09(f)2003].

Sample Preparation

Feulgen PAS stained monolayers of isolated nuclei were prepared as previously (Klanrit et al., 2007). Briefly, dysplastic areas highlighted on routine H&E sections were cut from paraffin-embedded blocks by scoring the block face with a scalpel before cutting at 50µm thickness. Sections of the selected area were deparaffinised in xylene, rehydrated in ethanols, suspended in PBS and nuclei digested from the cells in 0.05% protease type XXIV (Sigma UK) at 37^{o}C for 90 minutes with vigorous shaking. Large debris was separated with a 60µm nylon mesh filter, the nuclei washed by centrifugation, and dispersed monolayers made on microscope slides in a cytospin 4 centrifuge (Shandon UK), stained with Feulgen-Schiff and mounted below coverslips.

Image-based ploidy analysis

Each stained monolayer was scanned on two semiautomated DNA ploidy systems. The Fairfield system details are as previously published (Sperandio 2013). The system calculates the integrated optical density of each Feulgen stained nucleus at an imaging resolution of 170 nm/pixel with 10-bit grey scale resolution. The PWS system uses a Zeiss Axiocam MCM camera (Carl Zeiss micro imaging GmbH, Germany) with similar resolution of 162 nm/pixel and 10 bit grey resolution. Both systems use separate Windows PC-based programs to capture images and analyse data. Both systems were tested using the same Zeiss Axioplan II microscope and E-662 piezo autofocusing x40 objective (Physik

Instrumente GmbH & Co. KG, Germany) and H29XYZ motorised stage (Prior, UK).

Both systems sort nuclear images into galleries of epithelial, lymphocyte and fibroblast cell types. In the Fairfield system this was achieved using a manually created decision tree based on imaging parameters of nuclei of known type constructed in our laboratory from oral tissue samples. In the PWS software, sorting is performed using a machine learning algorithm of support vector machine system type.

Galleries of sorted nuclei were checked for sorting accuracy and cleaned by excluding cut and otherwise incomplete or overlapping nuclei by a biomedical scientist (CL). DNA ploidy histograms were constructed on both systems and reviewed and diagnosed by a pathologist (EWO). The number of nuclei of each type on completion was recorded together with the CV of the diploid and other defined peaks, and the 5c-exceeding rate. Diagnostic criteria were as previously validated and published for the Fairfield system (Danielsen et al., 2015; M Sperandio, 2013).

Statistical analyses

The distribution of the numbers of nuclei, 5c-exceeding rates and distribution of the differences in CV identified by the two systems were compared using quantile-quantile plots and kernel density plots. The distributions for cell count and 5C-exceeding rate were compared using the Wilcoxon signed-rank test and the medians compared using the sign test. As coefficients of variation cannot be directly compared, the differences in paired data were treated as a continuous variable. Normality of distribution was then tested using the omninorm test and the difference compared using the paired t test. The final ploidy diagnosis was compared between systems using the Spearman correlation test. All statistical calculations were performed on SPSS 23 and GraphPad Prism 6.0.

Results

The features of the lesions selected for this study by histopathological diagnosis, dysplasia grade and ploidy status are described in table 1.

Using the Fairfield system, 83 samples were diagnosed as diploid and 18 as aneuploid and in 11 cases diagnosis was deferred for insufficient nuclei in the sample. Using the PWS system, 91 cases were diagnosed as diploid and 19 as aneuploid and in 2 cases diagnosis was deferred for insufficient nuclei in the sample. In all cases, non-concordance was accounted for by the ability of the PWS system to capture data from cases classified as insufficient by the Fairfield system.

Both systems performed consistently without software errors or scanning failures. Quantile-quantile and kernel density plots showed that the underlying data distributions in the datasets obtained for total number of nuclei were different and the differences in distribution were significantly different (sign rank test $P<0.0001$) as were both central tendency and scatter (sign test $P<0.001$). The PWS system identified more nuclei than the Fairfield system (Table 2; Figure 1).

The distribution of differences between paired coefficients of variation was normal (ominorm test, $P=0.37$) and suitable for parametric analysis, which showed a significant difference between systems, the PWS system achieving smaller coefficients of variation for each diploid peak (2 sided t-test, $P<0.0001$; Table 2; Figure 2).

There was no difference between systems in detection of the 5c exceeding rate (Wilcoxon signed-rank test $P>0.54$) and the medians were the same (two-sided sign test $P=0.64$; Table 2).

Following analysis of the histograms, the final ploidy diagnosis was compared between the two systems and a strong correlation was found using the Spearman correlation test ($r=0.761$, 95%CI 0.72-0.89 and $p<0.0001$, Table 2). Ten cases (N=112) were classified into the indeterminate category by the

Fairfield system due to insufficient captured nuclei whereas the PWS system captured enough nuclei to establish a diagnosis. There was complete concordance in diagnosis between the two systems when cases with insufficient material were excluded.

Discussion

Applications for DNA ploidy analysis are constantly increasing (Danielsen et al., 2015) and flow cytometry and image based cytometry are the usual methods used. Although image-based cytometry has definite advantages in routine histopathological diagnosis, few systems are available commercially and many do not have sufficiently robust supporting data to be used in clinical diagnosis or achieve ISO15189 laboratory accreditation.

Data is already published for new clinical applications using the PWS system,(Mouradov, 2013; Pradhan et al., 2012; Pretorius et al., 2009) but it is not possible to constantly re-establish the clinical validity of new systems or software updates, because this requires expensive and time-consuming clinical outcome studies. Furthermore, reanalysing samples from previous studies may not be possible for technical reasons, including faded nuclear staining and insufficient material remaining in the paraffin blocks. It is therefore necessary to validate newer methods against those used in the studies that originally established sensitivity, specificity and predictive values.

Systems are not readily interchangeable without detailed analysis. Flow cytometry and image-based cytometry produce different results in epithelial tissue(Brouns et al., 2012) and slight differences in apparently similar image-based cytometry systems, which can affect predictive values. Both the Fairfield and ClearCyte (Perceptronix Medical, Vancouver, Canada) systems appear to work in a very similar fashion and accurately identify the highest risk cases, but differ in their ability to detect low risk cases.(Bradley et al., 2010; M Sperandio, 2013)

The present study provides a package of relatively simple statistical tests and testing parameters to establish equivalence between different methods or validate software upgrades. We compared the total number of cells collected for analysis, coefficient of variation of the diploid peak because this reflects

ability to distinguish peaks with close diploid indices, 5C exceeding rate because this is a diagnostic threshold for aneuploidy, and the final ploidy diagnosis.

Overall the PWS system produced comparable results to the Fairfield system, and shows some improvements consistent with its more recent camera and computer specifications. The PWS system captured more nuclei and, though not assessed formally, captured fewer duplicate images that had to be manually excluded during analysis. This is probably a result of collecting images from the centre of the cytospin sample in a spiral path, as opposed to the overlapping parallel scanning path of the Fairfield system. The PWS system also scanned and captured images more quickly, a reflection of its faster computer specification.

The quality of histograms for diagnosis was higher with the PWS system, with a lower and more consistent coefficient of variation of the diploid peak, well within the 5% required for diagnostic use. Subsequent routine use of the PWS system has shown that a CV of between 1 and 2% is readily achievable, giving an approximate resolution of a 1% change in DNA content per cell.(Danielsen et al., 2015)

The overall performance improvements of the PWS system lead to an ability to obtain a diagnosis in cases where the yield of nuclei is small and with higher resolution. There were no discordant diagnoses. We conclude that the PWS image cytometry DNA ploidy analysis system is comparable to the Fairfield system in terms of diagnostic outcome, and that its improved performance will be of value in a routine diagnostic laboratory.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

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Tables and figure legends

Table 1. Distribution of the sample lesions according to histological diagnosis, dysplasia grade and ploidy status.

Diagnosis	Dysplasia	n	Diploid (n)	Aneuploid (n)
CHC	none	9	9	0
Keratosis	none	21	21	0
Keratosis	mild	30	26	4
Keratosis	moderate	14	7	7
Keratosis	severe	8	2	6
LP/LR	none	25	22	1
OSMF	none	3	3	0
OSMF	moderate	1	1	0
SCC	NA	1	0	1
TOTAL		112		

Legend: CHC (chronic hyperplastic candidiasis), LP/LR (compatible with lichen planus or lichenoid reaction), OSMF (oral submucous fibrosis), SCC (squamous-cell carcinoma), NA (not applicable).
The PWS failed to capture enough nuclei for DNA ploidy classification in 2 samples, which were both LP/LR.

Table 2. Total nuclei identified, coefficient of variation of the diploid peak and 5C-exceeding rate obtained using the Fairfield (FF) and PWS systems.

	Total nuclei		Coefficient of Variation diploid peak		5c exceeding rate	
	FF	PWS	FF	PWS	FF	PWS
mean	760	1415	3.69	2.76	0.62	0.62
median	631	1529	3.49	2.61	0	0
min	50	165	0.86	1.41	0	0
maximum	2158	3201	7.49	5.63	15.97	15.91
95% ci low	681	1309	3.46	2.59	0.24	0.24
95% ci high	840	1521	3.91	2.92	0.99	0.98

Figure 1. Wilcoxon matched-pairs signed rank test for the number of nuclei captured by each system. The asterisk indicates a significant difference between the methods tested (P<0.0001).

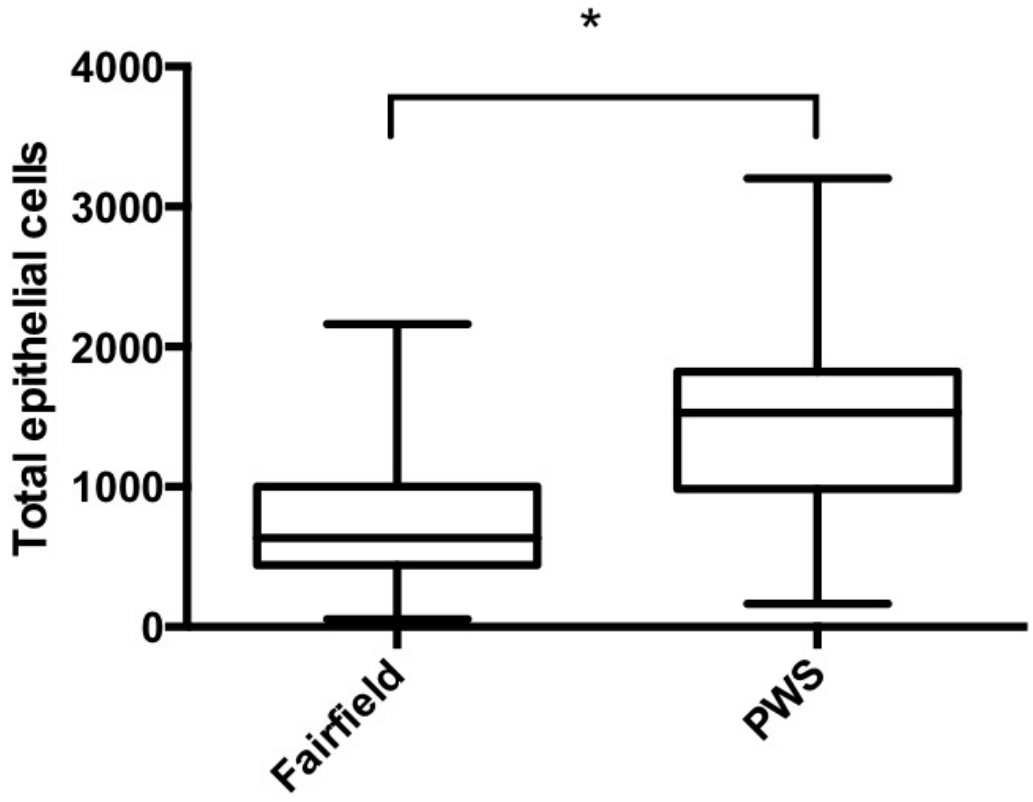

Figure 2. Paired t-test for CV. The asterisk indicates a significant difference between the methods tested (P<0.0001).